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# Influence of a polymerisable eugenol derivative on the antibacterial activity and wettability of a resin composite for intracanal post cementation and core build-up restoration

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## Abstract

**Objectives:** Eugenol has been used in dentistry due to its ability to inhibit the growth of a range of microorganisms, including facultative anaerobes commonly isolated from infected root canals. The aim of this study was to evaluate the antibacterial activity of the experimental composites containing eugenyl methacrylate monomer (EgMA), a polymeric derivative of eugenol, against a range of oral bacteria, commonly associated with failure of coronal and endodontic restorations. *In vitro* composite behaviour and wettability were also studied in conjunction with their antibacterial activity.

**Methods:** EgMA monomer (5 and 10% by weight) was added into BisGMA/TEGDMA resin based formulations with filler mixtures of hydroxyapatite (HA) and zirconium oxide ZrO<sub>2</sub>. The antibacterial activity of the experimental composites against *E. faecalis*, *S.mutans* and *P.acnes* were evaluated by direct contact test and compared with composite formulation without inclusion of EgMA. To clarify the antibacterial mode of action, agar diffusion test (ADT) was also performed.

Water sorption, solubility, diffusion coefficient, contact angle and surface free energy as complementary clinically relevant properties were determined.

**Results:** Water sorption and wettability studies showed reduction of water uptake and surface free energy values with increasing content of EgMA monomer, resulting in significant increase in the hydrophobicity of the composites. No inhibition zones were detected in any of the composites tested against the three bacteria employed as expected, due to the absence of any leachable antibacterial agent. The covalently anchored EgMA monomer with the composite surface exhibited an effective bacteriostatic activity by reducing the number of CFUs of the three species of bacteria tested with no significant dependence on the concentration of EgMA at 5 and 10% by weight. The surface antibacterial activity *R* of the experimental composites were different against the three tested species with values in the range 2.7 – 6.1 following the order *E. faecalis* < *S. mutans* < *P. acnes*.

**Significance:** The incorporation of EgMA monomer within polymerisable formulations provides a novel approach to yield intrinsically antibacterial resin composites for different dental applications.

**ALMAROOF, A., NIAZI, S. A., ROJO, L., MANNOCCI, F. & DEB, S. Influence of a polymerizable eugenol derivative on the antibacterial activity and wettability of a resin composite for intracanal post cementation and core build-up restoration. *Dental Materials*. doi:10.1016/j.dental.2016.04.001**

## 1. Introduction

Endodontically treated teeth (ETT) are more susceptible to fracture than vital teeth due to the significant reduction of tooth tissue as a result of the endodontic and restorative treatment accompanied with changes in chemical composition of dentine due to loss of water and collagen [1]. Although it is well established that coronal coverage significantly improves the clinical success rate of endodontically treated posterior teeth, the choice of restoration depends on the amount of remaining tooth structure and functional requirements.

Composite resins have the advantage of bonding to residual coronal and root canal dentine, which may assist in strengthening the tooth [2,3] and offer an alternative technique for restoration of ETT [4]. Dual cure resin composites core materials with different viscosity are currently used within the canal for fibre posts cementation, to restore the structurally compromised ETT. They have superior mechanical properties than those of resin cements and result in lower stress, reducing the load transfer on the root dentine and surface of the post [2].

However, resin composites lack antibacterial properties and result in more plaque accumulation than other restorative materials [5]. In addition, any microleakage allows for new bacterial invasion, compounded by the fact that it is difficult to completely remove bacteria from the root canal system even after careful cleaning and shaping and the minimally invasive approach during restoration of teeth will possibly maintain more residual bacteria within the dentinal tubules [6].

There is a rising interest to endow dental restorative materials with sustained antibacterial activity to enhance long term performance [7], which is expected to lower the risk of reinfection [8] and secondary caries [9]. Different antibacterial

agents such as chlorhexidine, fluoride, quaternary ammonium salts and metallic agents (silver, gold and zinc) have been incorporated in acrylic based composite formulation in order to achieve this goal [10]. However, most of these additives cause an adverse effect in terms of mechanical properties, discoloration of the material [11], toxicity and short-term antibacterial effectiveness [12].

Most antibacterial studies reported in literature evaluate the activity of different incorporated antibacterial agents against *Streptococcus mutans*, the main microbial etiological agent of dental caries and the leading cause of resin based composite failure [13], however other oral microorganisms such as, *Enterococcus faecalis*, *Candida albicans* and *Propionibacterium acnes* are also frequently associated with endodontic infections [14]. *E. faecalis*, in particular, is difficult to remove owing to its considerable virulence factors constituting a source of recurrent infection after conservative as well as surgical treatments [15]. *P. acnes* is an anaerobic Gram-positive bacterium responsible for a wide range of infections and inflammatory conditions [16]. Therefore, development of antibacterial restorative filling materials to be reliable for a variety of dental applications need a potent antimicrobial agent which acts against a wide range of oral microorganisms.

Eugenol (4-allyl-2-methoxyphenol) is a natural phenolic anti-oxidant essential oil that possesses antifungal activity [17] and inhibits the growth of several microorganisms including *Escherichia coli* [18] and facultative anaerobes commonly isolated from infected root canals [19]. This compound has been used in combination with zinc oxide in different dental applications such as temporary filling materials and root canal sealers. However, eugenol is not compatible with other methacrylate based restorative materials because of the presence of free eugenol, which interferes with the polymerisation reaction of dental composite resins.

In contrast, eugenyl methacrylate (EgMA) an eugenol derivative [20] possess in its chemical structure a polymerisable methacrylic group (Fig 1) that allows the monomer to participate in free radical polymerisation reactions whilst maintaining the antibacterial activity of its natural precursor against different Gram-negative and Gram-positive bacterial species [21].

In our previous study, the experimental composites from Bis-GMA/TEGDMA, a commonly used dental resin system and EgMA were formulated with 65 % by weight filler phase comprising of HA/ZrO<sub>2</sub> [22]. These composites were tailored to function as an antibacterial restorative material for intracanal posts cementation and core build-up in the restoration of ETT. The influence of EgMA monomer incorporation on curing, physical and mechanical properties of these new formulations showed that these composites were suited for the application.

However, properties such as water sorption and wettability have detrimental effects on the composite material and bacterial adhesion [23] which are important parameters toward clinical relevance. Hence in this study, the *in vitro* behaviour and antibacterial activity of these EgMA containing resin composites against a range of oral bacteria commonly associated with the failure of coronal and endodontic restorations are reported.

## **2. Materials and Methods**

### **2.1. Materials and composites formulations**

Three batches of dual cure resin composites were prepared by combining 2, 2-Bis [4- (2-hydroxy-3 methacryloyloxypropyl)-phenyl] propane (Bis-GMA) (Esschem Europe, Durham, UK) and tri-ethyleneglycol dimethacrylate (TEGDMA) (Esschem Europe, Durham, UK) in a fixed ratio of 1:1 by weight, representing a total resin phase of 35 wt % in the formulation. EgMA monomer (MW = 232.23 g/mol.) was synthesised as reported previously [20] and added at a level of 0 (reference), 5 and 10 wt. % of the resin phase (Table 1). All composites were formulated with 65 wt. % filler phase, which contained hydroxyapatite (HA) with a mean particle size diameter of 3-5  $\mu\text{m}$  (Plasma Biotall Ltd., Tideswell, Derbyshire, UK) and  $\text{ZrO}_2$  with a mean particle size diameter 18  $\mu\text{m}$  (Fisher Scientific Ltd., Loughborough, UK) in a ratio of 4:3 by weight. The filler particles were silanated with 10% of A-174 (Merck-Frankfurt, Germany) by a wet silanation treatment in 70/30 mix of acetone and distilled water following a method described previously [24]. The resin phase was first prepared and divided in two separate portions where initiator system (0.5 % benzoyl peroxide (Merck-Frankfurt, Germany) + 0.5% camphorquinone (Sigma-Aldrich, Dorset, UK)) and activator (N,N dimethyl p-toluidine (Sigma-Aldrich, Dorset, UK) 1:1 molar ratio) were added respectively to avoid self-polymerisation. Then the corresponding amount of silanised filler was added to each portion and mixed on a magnetic stirrer for 24h.

### **2.2. Sample preparation**

Equal masses of the two pastes were hand-mixed using a stainless steel spatula for 30 seconds and carefully placed into Teflon moulds to produce discs of 10 mm

diameter and 1 mm thickness avoiding bubble entrapment. The upper and lower surface of the mould was covered with glass slides and then cured by visible light for 40 s each side by overlapping, using Optilux 501 (Demetron, Danbury, U.S.A.) dental curing unit performing an irradiance of  $400 \pm 50 \text{ mW cm}^{-2}$ .

### **2.3. Water sorption and solubility**

Water sorption and solubility were measured according to ISO 4049 [25]. Three disc specimens were prepared for each material. The thickness and diameter of each specimen were measured at 4 and 2 points respectively, using a digital electronic caliper (DURATOOL, UK). Mean values were used to calculate the volume of each specimen in  $\text{mm}^3$ . The specimens were then placed in a desiccator with anhydrous calcium chloride and maintained at  $37^\circ\text{C}$ . After 22h, they were removed, stored in another desiccator at  $23^\circ\text{C}$  for 2h and then weighted to an accuracy of  $\pm 0.0001 \text{ g}$  using a Mettler-Toledo AG64 balance to obtain the constant initial weight ( $M_i$ ) and to ensure completion of polymerisation and dehydration. Specimens of each material were immersed in 10 ml distilled water in individual glass containers and then incubated at  $37^\circ\text{C}$  for a total immersion time of 28 days. At noted intervals, the specimens were gently dried on filter paper until free from visible moisture, waved in air for 15 s and weighed 1 min later and returned to the glass containers filled with distilled water. The recorded weight was denoted as the mass of saturated specimen  $M_s(t, \text{time})$ . Each specimen was then desorbed in a drying oven maintained at  $37^\circ\text{C}$  and weighed again until a constant dry mass ( $M_d$ ) was found. A second absorption–desorption cycle followed to obtain  $2^{\text{nd}} M_s(t, \text{time})$  and  $2^{\text{nd}} M_d$  in the same way as the first cycle.

Mass change percentage was calculated by the following equations:



$$\text{Sorption mass change percentages} = (M_{s(t)} - M_i)/M_i \times 100 \quad (1)$$

The water sorption ( $W_{SP}$ ) and solubility ( $W_{SL}$ ) in  $\mu\text{g} / \text{mm}^3$  were calculated using the following equations:

$$W_{SP} = M_s - M_d / V \quad (2)$$

$$W_{SL} = M_i - M_d / V \quad (3)$$

Where,  $V$  is the volume of the sample.

The early stages of diffusion-controlled uptake of water in composites are given by

$$Mt/M^\infty = 2 \left( \frac{Dt}{\pi l^2} \right)^{1/2} \quad (4)$$

where,  $Mt$  is the mass uptake at time  $t$ ,  $M^\infty$  is the equilibrium uptake,  $l$  is the thickness, and  $D$  is the diffusion coefficient.

Diffusion coefficients were evaluated from the slope values of the initial linear part of the sorption curves. A plot of  $Mt/M^\infty$  against  $t^{1/2}$  should provide a straight line with the slope,  $s$ , then given by

$$s = 2 \left( \frac{D}{\pi l^2} \right)^{1/2} \quad (5)$$

and the value of  $D$  calculated from the slope value.

$$D = \frac{s^2 \pi l^2}{4} \quad (6)$$

## 2.4. Measurement of contact angle and surface free energy (SFE)

The contact angle  $\theta$  and SFE ( $Y_s$ ) were determined on composites surface discs using the sessile drop method. Ten 5  $\mu$ l droplets of two liquids with opposite polarity and known surface tension were placed: water ( $Y_l$ ) 72.8 mN/m<sup>2</sup> and methylene iodide ( $Y_l$ ) 51.8 mN/m<sup>2</sup>.

$Y_l$  refers to the total surface free energy of the liquid. The contact angle was then measured at room temperature 20s after drop placement by imaging the drop with a magnified digital camera. The profile of the drop was then processed with ImageJ software. The surface free energy ( $Y_s$ ) of the composites was calculated by the Fowkes' [26] and Owens' [27] method.

$$Y_s = Y_s^d + Y_s^p \quad (7)$$

$$(1 + \cos \theta) Y_l / 2 = (Y_s^d Y_l^d)^{1/2} + (Y_s^p Y_l^p)^{1/2} \quad (8)$$

where  $Y_s^d$ ,  $Y_s^p$ ,  $Y_l^d$ , and  $Y_l^p$  are the dispersive and polar components of  $Y_s$  of solid and liquid, respectively. The polar and dispersive values for the tested liquids were taken from the literature [28].

## 2.5. Antibacterial assay

### 2.5.1. Agar Diffusion Test

Antibacterial activity of the composites against *Enterococcus faecalis*, *Streptococcus mutans* and *Propionibacterium acnes* was determined by a standard Agar Diffusion Test (ADT). All discs were sterilised by wiping with 70% ethanol in water and were exposed to UV radiation for 30 mins. The bacteria were evenly spread onto the

Fastidious Anaerobic Agar (FAA, Lab M, UK) supplemented with 5% defibrinated horse blood. Under aseptic conditions, 5 discs of each of the test composite (0% EgMA, 5% EgMA, 10% EgMA) were placed onto these FAA plates (one disc for each plate). The plates without discs (n=2) of each bacteria species were used as controls. All plates were incubated anaerobically at 37 °C. The inhibition zones around each specimen were checked after 48h and again after 4 days.

### **2.5.2. Measurement of antibacterial activity on composite surfaces**

The antibacterial activity of the composites surface were evaluated *in vitro* against the adherence and growth of *E. faecalis*, *S. mutans* and *P. acnes* following an adapted protocol from ISO 22196:2007 standard for the measurement of antibacterial activity on plastic surfaces [29].

All discs were sterilised by wiping with 70% ethanol in water and were exposed to UV radiation for 30 min. For each bacterial species, the test was performed on 4 discs of each of the test composites (with 5% EgMA, 10% EgMA) and 8 discs of the control group with no additive (0% EgMA). Half of the control group (4 discs for each bacterial species) were measured for the colony forming units (CFUs) immediately after inoculation and the other half were measured after incubation for 24h.

The discs were placed in a separate well within a sterile 16-wells plate (Corning®, NY, USA). *E. faecalis*, *S. mutans* and *P. acnes* were cultured anaerobically at 37°C overnight on FAA plates (FAA, Lab M, UK) supplemented with 5% defibrinated horse blood. One loopful of the bacterial culture was inoculated into 100µl of brain heart infusion (BHI) broth, serially diluted to obtain the selected optical density having bacterial concentration of  $10^6$  CFUs/ml. 150µl of the bacteria suspension was pipetted onto the disc surface so that it stays onto the surface and does not leak

beyond the edges of the disc. Before incubating the discs anaerobically, half of the control discs were washed with 850µl of sterile Phosphate Buffered Saline (PBS). To ensure that PBS completely washes the discs, the same PBS was collected and pipetted four times. Finally the collected PBS was serially diluted in BHI, plated in duplicates onto FAA plates and incubated anaerobically. The number of colonies was counted after 48h and repeated again after 4 days. The plates with the other discs (other half of the control, 5% and 10% EgMA composites) were incubated anaerobically at 37°C for 24h. After incubation they were washed with sterile PBS, the collected PBS was serially diluted, plated in duplicates onto FAA plates and incubated anaerobically for quantitative viable counts as mentioned above.

The number of viable bacteria recovered was determined according to the following equation:

$$N = \frac{C \cdot D}{A} \quad (9)$$

where,

$N$  is the number of viable bacteria recovered per  $\text{cm}^2$  per test specimen;

$C$  is the average plate count for the duplicate plates;

$D$  is the dilution factor the plates counted;

$A$  is the surface area of test specimen in  $\text{cm}^2$ .

The antibacterial activity  $R$  was calculated using the following equation:

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \quad (10)$$

where,  $R$  is the antibacterial activity;  $U_0$  is the average of the logarithm of the number of viable bacteria, in cells·cm<sup>-2</sup>, recovered from the control specimens immediately after inoculation;  $Ut$  is the average of the logarithm of the number of viable bacteria, in cells cm<sup>-2</sup>, recovered from the control test specimens after 24h;  $At$  is the average of the logarithm of the number of viable bacteria, in cells cm<sup>-2</sup>, recovered from the EgMA containing test specimens after 24h.

## 2.6. Statistical analysis

The mean values of water sorption, solubility,  $D$ ,  $\theta$ ,  $Y_s$  and logarithmic CFU count / cm<sup>2</sup> were calculated and differences was analysed by one-way ANOVA, followed by Tukey's post hoc analysis at significance level of  $p < 0.05$ . Independent-samples T test was used to compare the antibacterial activity ( $R$ ) of composites between two different concentrations of EgMA. Standard deviation (SD) of each experiment were calculated and represented in brackets alongside the respective mean value.

## 3. Results

### 3.1. Water sorption and solubility

Plots of  $Mt/M^\infty$  versus  $t^{1/2}$  for the composites tested during water sorption in the first and second cycle are shown in Fig 2. All curves were fit to a linear regression during the early stages of diffusion-controlled uptake of water; which allows the calculation of the diffusion coefficients from the initial curve slope. The water uptake weight percentage, water sorption, solubility values and the sorption diffusion coefficient of the experimental composites during the first and the second cycle are summarised in Figure 3 and Table 2. In both cycles, all composites reached equilibrium within the first week of immersion; the water sorption and diffusion coefficients values were significantly reduced with increasing content of EgMA ( $p < 0.05$ ). The values of the

diffusion coefficients calculated were larger during second sorption cycle. For water solubility, although slight reduction was observed for EgMA containing composites, the statistical analysis showed significant reduction in the solubility value of BTEg10 composite only during the second cycle when compared with the control (BTEg0) composite.

### **3.2. Wettability of the composites**

The contact angle ( $\theta$ ) values and surface free energy ( $\gamma_s$ ) are summarised in Table 3. The addition of EgMA in the composite formulation significantly increased the contact angle measurements with both tested liquids which resulted in a significant reduction of the calculated surface free energy ( $p < 0.05$ ), indicating the higher hydrophobicity of composites surfaces.

### **3.3. Antibacterial assay**

#### **3.3.1. Agar Diffusion Test**

The results of this test showed that the lack of inhibition zone detected around the specimens of the 3 test composites against the 3 bacteria tested (*E. faecalis*, *S. mutans* and *P. acnes*), indicating that there was no elution of any antibacterial component from the bulk specimens.

#### **3.3.2. Antibacterial activity of the composites surface**

Figure 4 illustrates the respective plate images of *E. faecalis*, *S. mutans* and *P. acnes* strains at dilution factor (-2) and after 24h incubation on composite surfaces according to the ISO 22196:2007.

The number of bacteria as  $\log_{10}$  CFU per test composite and the calculated *R* values representing the antibacterial activity of composites are shown in Figure 5 and Table

4 respectively. From the results, it can be seen that, for control group the log<sub>10</sub> CFU recovered were significantly higher than those recovered immediately after inoculation ( $p < 0.01$ ). The number of colonies of all tested bacteria was reduced by the addition of EgMA into the formulation of the composites and the log<sub>10</sub> CFU of these composites were significantly lower as compared with the control composites ( $p < 0.01$ ). No significant difference was observed in antibacterial activity between BTEg5 and BTEg10 composites ( $p > 0.05$ ) with  $R$  values of the antibacterial activity  $R$  ranged between 2.7 and 6.1 following the order *E. faecalis* < *S. mutans* < *P. acnes*.

#### **4. Discussion**

In present study, the new composite formulations bearing the eugenyl derivative monomer showed an effective bacteriostatic activity against different oral microorganisms. The novelty of these formulations originates from the immobilisation of eugenol molecule within the resin matrix, preventing its potential side effect against surrounding tissue and enhancing the hydrolytic stability, while maintaining the biological properties of eugenol.

The inhibitory effect of eugenol against a variety of oral bacteria has been reported [19], however, one of major limitations of eugenol applications in dentistry is derived from the antioxidant character of the unreacted molecules of eugenol which inhibit the free radical polymerisation of dental composite resin materials and can also produce tissue irritation. By modifying the chemical structure of eugenol, EgMA monomer was synthesised after acylation reaction of eugenol with methacrylic chloride, the new derivative has the ability to copolymerise with other methacrylate monomers and immobilise the antibacterial eugenol moieties in the polymer

backbone without the inhibitory effect characteristic of the phenol derivatives [30]. Moreover, cytocompatibility of EgMA containing polymer matrices has been reported earlier with the absence of any toxic eluants [20].

The use of EgMA monomer in the formulation of the experimental composites offers several advantages in terms of complete miscibility with Bis-GMA/TEGDMA mixtures, improved mechanical properties and better processability and handleability of the corresponding composite pastes [22]. In addition, the immobilisation of the antimicrobial agents offers an additional benefit in comparison with other systems where the mode of action involve leachable antimicrobial agents such as chlorhexidine, which lacks the miscibility with other dental monomers and thus leading to adverse influence on mechanical properties, increased water sorption, porous structure and short term effectiveness [31].

Water sorption and solubility of polymeric composites are of importance for dental applications. The physical and mechanical properties of resin composite materials may be significantly altered by the effects of water sorption and component elution. Fluid uptake in an oral environment also leads to harbouring of bacteria within these composites that eventually lead to discolouration and failure. Several factors, including monomers hydrophilicity, cross-link density and the presence of fillers can affect the water sorption of resin based composite materials [32].

The water uptake of the experimental formulations with and without EgMA showed values that were in agreement with previous data on commercial composite core formulations [33,34] and fulfilled the requirements for dental applications in accordance to ISO 4090 standard requirements that limit the values of water sorption and solubility to a maximum of  $40 \mu\text{g}/\text{mm}^3$  and  $7.5 \mu\text{g}/\text{mm}^3$  respectively. The



reduction in water sorption and diffusion coefficients values with increasing content of EgMA monomer was due to monomer hydrophobicity and ability to form slightly cross linked structures [35] which reduced the water permeability of the polymer by decreasing the free space and thereby the swelling of the polymer. This finding was consistent with other studies in which EgMA was copolymerised with other methacrylate monomers, ethyl methacrylate (EMA) [35] and 2-hydroxyethyl methacrylate (HEMA) [21]. It has been reported also that the incorporation of HA fillers reduced the water uptake of methacrylate based dental composites especially when the HA particles were surface treated with a silane coupling agent [36]. Therefore, the use of HA particles within our experimental formulations had a distinct contribution in lowering water uptake, limiting the extraction of unreacted components which cause weight loss and adversely effects mechanical properties and longevity of these materials [37].

The water uptake indicates that the sorption behaviour of the composites follow Fickian diffusion and the coefficients of diffusion obtained in this study were comparable to published values obtained from resin composites formulated with silanised HA filler [38] with higher values of the diffusion coefficients for second sorption cycles (Table 2). This trend was also observed for conventional resin composites in previous studies [38,39] and can be explained by the fact that the movement of the water molecules in the first sorption is hindered from the eluting monomer molecules. The water sorption values were also higher in the second sorption cycle due to the loss of unreacted components and the solubility of the experimental composites correlated with their water sorption behaviour as expected.

The contact angle and surface free energy are important parameters for determining the hydrophobicity of the materials and their interactions with medium and usually

related with its water sorption. There is an effect of SFE substrates on bacterial adhesion, which has been critically discussed in the literature, however there is no clear consensus. Bacterial adhesion is a complex phenomenon and is related to the surface energy of a solid. However, this relationship is not a linear correlation since the chemical composition of the surface tends to govern the interaction which depends on both the chemistry of the solid and immersion liquid and additionally the type of bacteria and growing media. This has resulted in conflicting reports with reports suggesting that materials with low SFE result in less bacterial adherence [23,40]; whilst other contrary reports found that bacterial adhesion decreased with increasing surface energy of substrates [41-43]. The addition of EgMA monomer containing a substituted aromatic ring into the formulation significantly increased the hydrophobicity leading to an increase of the surface contact angle and reducing the SFE values from 54.3 mN/ m for the control, to 47.7 and 44.8 mN/ m for composites containing 5% and 10% EgMA respectively. The antibacterial activity of the experimental composites is derived from the pendent eugenyl residue from polymer network (Fig 1). Therefore, the ability of these composites to reduce or inhibit bacterial growth is highly dependent on the direct contact between eugenyl residues of the composite surface and the bacteria.

According to the bacterial adhesion theory DLVO [44], the total interaction energy between the bacteria and the solid surfaces is the sum of several interaction components such as Van der Waals attractive interaction, electrostatic double-layer repulsive component, Lewis acid–base component. These interactions are entirely related to the SFE of substrates. It was noted that, the greater hydrophobic character of EgMA containing composites, presented a lower SFE, improved the total interaction energy with the bacteria and resulted in a higher accessibility to the

eugenyl moieties responsible for the bacteriostatic activity. This could explain the reduction in number of CFU of bacteria tested and is in agreement with other studies which found that the total interaction energy between the bacteria and the substrate is linearly increased with the decrease of the surface energy [23,41].

Agar diffusion test findings were consistent with other studies on bactericide-immobilised materials, such as zinc oxide nanoparticles [45] and methacryloyloxydodecyl pyrimidinium bromide (MDPB) [46], incorporated into resin composite with no leachable antibacterial agents exhibiting surface inhibition properties. The lack of inhibition zones around composites discs containing EgMA confirmed their non-releasing behaviour as the inhibition zones can only be formed by the diffusion of antimicrobial material indicating that the antibacterial activity of the composites is not through the release of agent to the medium but is associated with surface contact.

The surface antibacterial activity test is a standardised quantitative assessment of the inhibitory effect on bacteria that contact the composite surface on which the EgMA is immobilised. It is clearly evident from the results that composites with eugenyl moieties had a highly significant inhibitory effect on the three types of bacteria tested, indicating that the chemically bound monomer has the capability to reduce or inhibit the colonisation of these bacteria which come into contact with composites surfaces. The significant differences between the numbers of CFU of the control samples recovered immediately and after 24h incubation indicating the favourable incubation growth condition for the bacteria and confirming the validity of the test. Composite containing 10% EgMA showed the highest inhibitory effect and reduced the number of *S.mutans* and *P. acnes* colony more efficiently than 5% EgMA composite. However, no significant difference was found between the two

concentrations for all tested bacteria. As mentioned above the effectiveness of the composites depend mainly on the contact between the eugenyl residue of the monomer pendent from polymer network and the bacteria. Therefore, the amount of these residues distributed on the surface of the sample may not differ greatly by increasing the monomer content from 5 to 10 wt %.

The exact mechanism of the bacteriostatic properties of immobilised eugenol containing materials remains unclear. However, eugenol below its minimum inhibitory concentration (sub-MIC) has been demonstrated to reduce the virulence properties such as adherence and biofilm formation of the cariogenic bacteria [47, 48]. In addition other studies have demonstrated the effect of eugenol on a variety of oral bacteria causing disruption in cell membrane permeability [49] and proton pumps [50] that reduce microbial resistance and biofilm formation on dental materials, which explains the well-known bacteriostatic properties shown in other eugenol containing materials commonly used in dentistry such as zinc oxide eugenol cements and eugenol based root canal sealers [51].

The value of the antibacterial activity  $R$  of EgMA composites in this study was higher than 2.0, the minimum accepted value for materials to exhibit an effective antibacterial surface [52]. The difference in  $R$  values against the three tested types of bacteria can be attributed to respective virulence of each species and to the differences in the chemical composition and structure of the bacteria cell walls that resulted in different bacterial sensitivities toward EgMA.

Lower  $R$  values achieved against *E. faecalis*, a facultative anaerobe, which is one of the resistant bacteria commonly found in the root canals of teeth with endodontic treatment failure [53]. *E. faecalis* has displayed resistance to a wide range of

antibiotics [15]. Moreover, it has been found that the effective proton pump mechanism which maintains optimal cytoplasmic pH levels of these species may participate in their resistance to the antimicrobial effects of calcium hydroxide [54].

*S. Mutans* virulence factors include its ability to synthesise adhesive glucans [55] and generate acids that result in the demineralisation of dental tissues, thereby initiating dental caries. Studies in literature reported that eugenol can effectively suppress the virulence of *S. mutans in vitro* by reducing the total mass of microorganisms and by virtue of its anti-adherence against this bacterium [56]. Eugenol at sub-MIC concentrations has the ability to inhibit the formation of adhesive glucans synthesised by glucosyltransferases (GTFs) that provide specific binding sites for bacterial colonisation on the tooth surface and binding to each other [55].

The testing of the antibacterial activity of the composite showed an excellent performance against *P. acnes* reported the highest *R* values. *P. acnes*, an opportunistic pathogen in refractory endodontic infection, may be associated with contamination of dental materials during the root canal filling procedure and has been isolated from infected root canals [16]. This species has been found to have high sensitivity to essential oil components that may adhere to the bacterial surface at low concentration [57].

## **5. Conclusion**

The incorporation of EgMA monomer as immobilised bactericidal moieties within polymerisable formulations provides a novel approach to develop resin composite materials with intrinsically antibacterial activity against oral bacteria commonly associated with coronal and endodontic restorations failures and therefore indicating their potential for use in different clinical applications.

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**Table 1. Composition of the resin phase of the experimental composites**

Composites		Monomers (in weight percent)		
Name		BisGMA	TEGDMA	EgMA
BTEg0		17.5	17.5	0
BTEg5		15.0	15.0	5
BTEg10		12.5	12.5	10

All composite formulations contained the same 65 % by weight filler phase of HA/ZrO<sub>2</sub> (4:3, wt/wt). The initiators (0.5 % benzoyl peroxide + 0.5% camphorquinone ) and activator (N,N dimethyl p-toluidine 1:1 molar ratio) were added as wt.% in respect to monomer at the end of the final resin monomers blend (100 wt.%) formulation.

**Table 2 – Mean (SD) values of water sorption, solubility and sorption diffusion coefficient for composite materials, for a total immersion period of 28 days.**

Composites	Water sorption ( $\mu\text{g}/\text{mm}^3$ )	Water solubility ( $\mu\text{g}/\text{mm}^3$ )	Diffusion coefficient ( $10^{-8} \text{ cm}^2 \text{ s}^{-1}$ )
<b>BTEg0</b>			
1 <sup>st</sup> cycle	34.8 (0.5)	4.6 (0.8)	2.87 (0.02)
2 <sup>nd</sup> cycle	39.0 (0.8)	2.3 (0.4)	4.08 (0.03)
<b>BTEg5</b>			
1 <sup>st</sup> cycle	27.1 (0.8)*	4.5 (0.3)	2.21 (0.03)*
2 <sup>nd</sup> cycle	29.7 (0.7)**	1.5 (0.7)	3.26 (0.01)**
<b>BTEg10</b>			
1 <sup>st</sup> cycle	20.4 (0.8)*	4.2 (0.3)	1.62 (0.01)*
2 <sup>nd</sup> cycle	21.1 (0.2)**	1.1 (0.5)**	2.89 (0.02)**
Differences were statistically significant with respect to control BTEg0 composite (* 1st cycle, ** 2nd cycle) (p < 0.05).			

**Table 3. Contact angle and solid surface free energy components for the experimental composites.**

Composites	$\theta$ (H <sub>2</sub> O)	$\theta$ (CH <sub>2</sub> I <sub>2</sub> )	$Y_s$ (mN/m)	$Y_s^d$ (mN/m)	$Y_s^p$ (mN/m)
BTEg0	54.9(2.9)	43.2(1.6)	54.3 (1.9)	38.6	15.6
BTEg5	64.7(3.5)*	46.8(3.3)*	47.7 (1.2)*	36.7	11
BTEg10	68.8(3.4)*	49 (2.5)*	44.8 (1.7)*	35.5	9.3

$\theta$  (H<sub>2</sub>O) = water contact angle;  $\theta$  (CH<sub>2</sub>I<sub>2</sub>) = methylene iodide contact angle; and  $Y_s$  = surface free energies with their dispersive ( $Y_s^d$ ) and polar ( $Y_s^p$ ) components.  
 \* Differences were statistically significant with respect to control BTEg0 composite (p < 0.05).

**Table 4 Antibacterial activity (*R*) of experimental composites tested according to the ISO 221 96:2007 after 24h incubation.**

	<i>E. faecalis</i>		<i>S.mutans</i>		<i>P. acnes</i>	
	<i>N</i>	$R = U_t - A_t$	<i>N</i>	$R = U_t - A_t$	<i>N</i>	$R = U_t - A_t$
	(CFU/cm <sup>2</sup> )	(log <sub>10</sub> CFU)	(CFU/cm <sup>2</sup> )	(log <sub>10</sub> CFU)	(CFU/cm <sup>2</sup> )	(log <sub>10</sub> CFU)
BTEg0	1.3 X10 <sup>8</sup>	U <sub>t</sub> = 8.1	6.8X10 <sup>7</sup>	U <sub>t</sub> = 7.8	5.8X10 <sup>7</sup>	U <sub>t</sub> = 7.7
BTEg5	3.7X10 <sup>5</sup> *	2.7	2.3X10 <sup>3</sup> *	4.6	1.1X10 <sup>2</sup> *	5.7
BTEg10	2.2X10 <sup>5</sup> *	2.8	1.0X10 <sup>3</sup> *	4.9	4.7X10 <sup>1</sup> *	6.1

*N*: the mean number of viable bacteria recovered per cm<sup>2</sup>; *U<sub>t</sub>*: is the average of the logarithm of the number of viable bacteria, in cells·cm<sup>-2</sup>, recovered from the control test specimens after 24h; *A<sub>t</sub>*: is the average of the logarithm of the number of viable bacteria, in cells·cm<sup>-2</sup>, recovered from the EgMA containing test specimens after 24h. \* Significant differences with respect to control BTEg0 composite (p < 0.01).

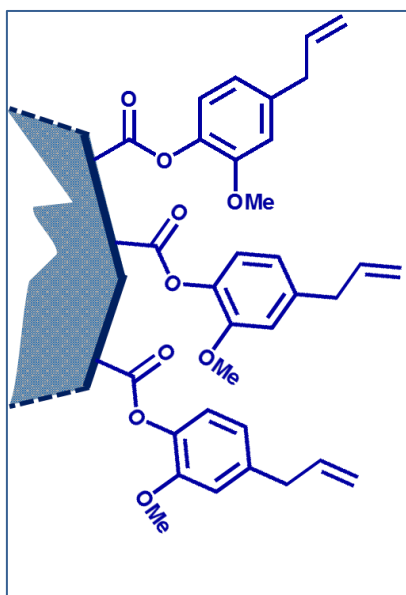




Figure 1. Proposed example of pendent eugenyl residues (eugenyl methacrylate antibacterial monomer) from the polymer network on the surface of the experimental composite.

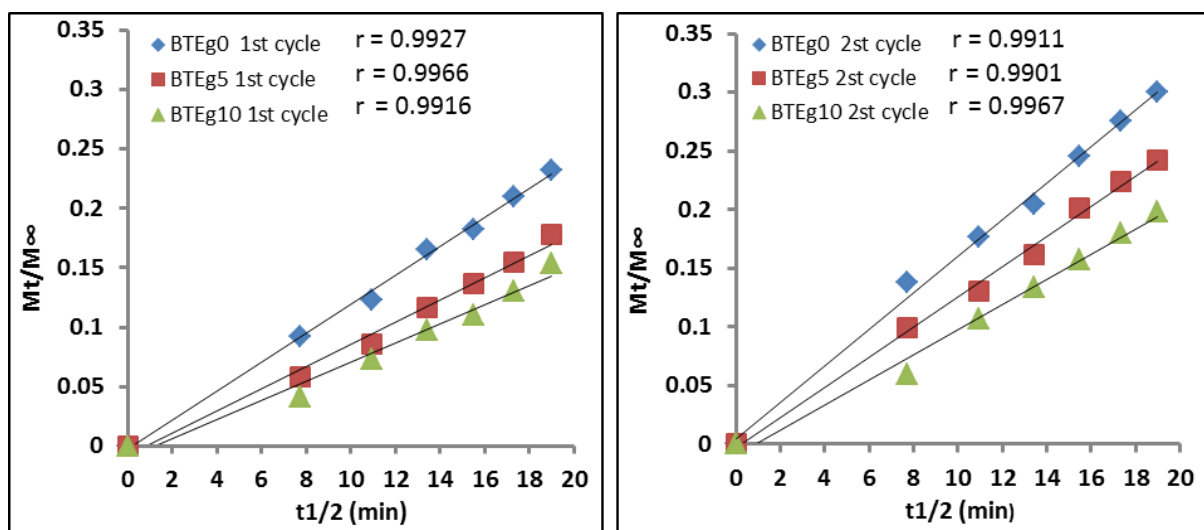


Figure 2. Early water uptake behaviour and linear fit of the composites during the first and second sorption cycle.

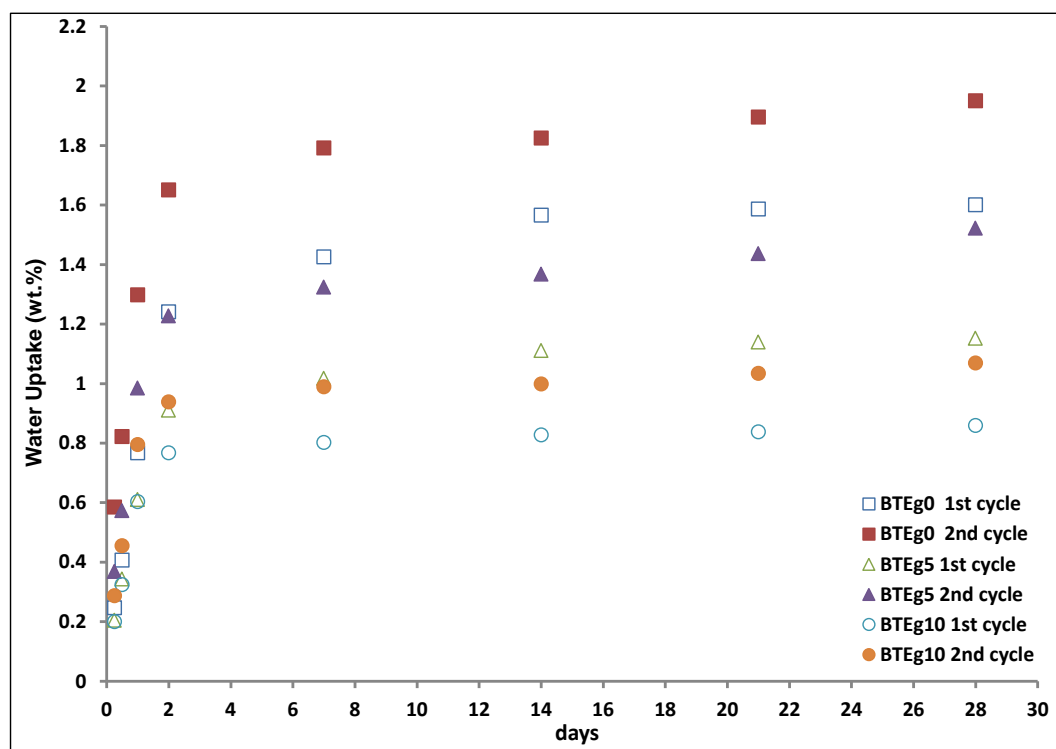


Figure 3. Mass change in percentage of the composites during immersion in water over 28 days. (First and second sorption cycle).






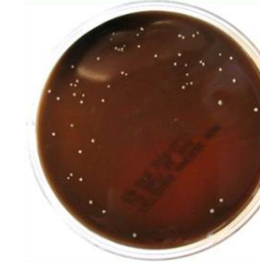



Strain	BTEg0	BTEg5	BTEg10
<i>E. faecalis</i>			
<i>S. mutans</i>			
<i>P. acnes</i>			

Figure 4. Respective plate images of composites surface antibacterial activity assay according to the ISO 22196:2007 after 24h incubation (dilution factor -2).

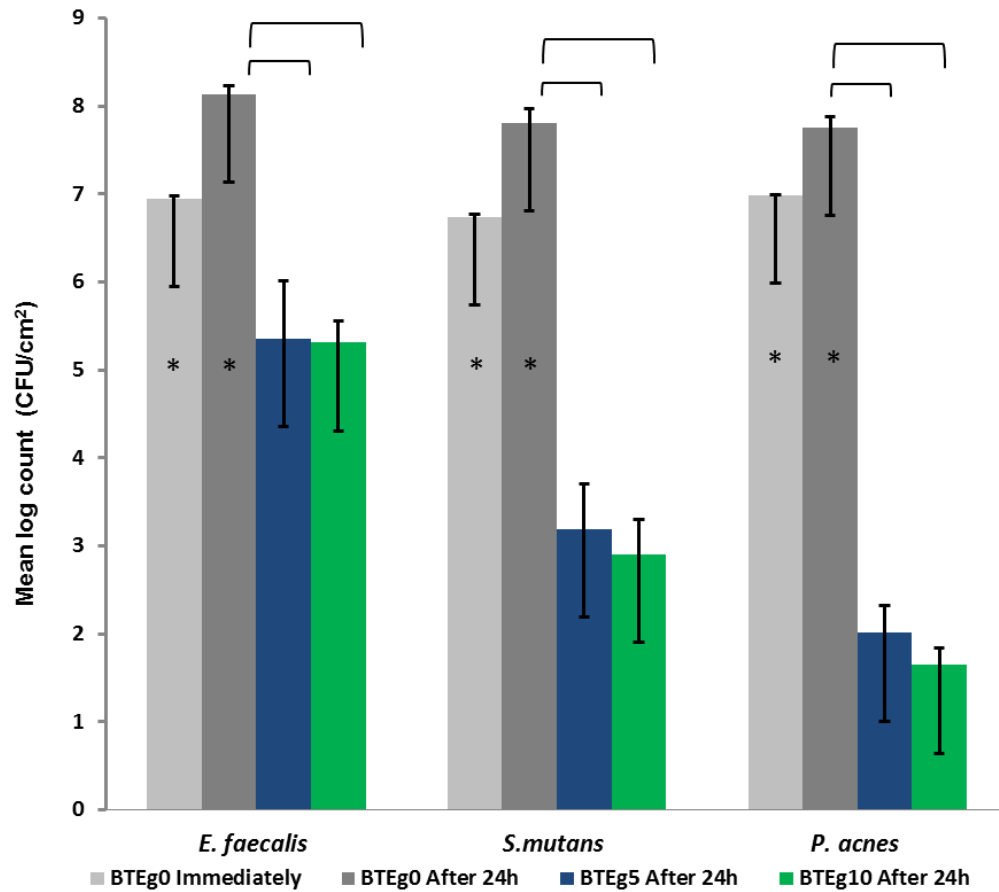


Figure 5. The mean  $\log_{10}$  CFU /  $\text{cm}^2$  of tested bacterial species on the experimental composites. \* indicates significant differences between control groups (BTEg0) recovered immediate and after 24 h incubation ( $p < 0.01$ ). The connection with horizontal lines indicates significant differences between groups after 24 h incubation ( $p < 0.01$ ).